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Long-Term Survival of Intestinal Allografts Induced by Costimulation Blockade, Busulfan and Donor Bone Marrow Infusion

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Tolerance-inducing strategies that infuse donor bone marrow cells in conjunction with costimulation blockade have not been applied to intestinal transplantation. Intestines from BALB/c mice were transplanted into C57BL/6 recipients treated with anti-CD40L mAb, CTLA4-Ig, donor bone marrow, and busulfan. The majority of mice transplanted after completion of this regimen developed hematopoietic macrochimerism, although the degree of chimerism varied widely between recipients, and experienced long-term allograft survival. T cells from these mice demonstrated donor-specific hyporesponsiveness *in vitro*. However, T cells from chimeric mice proliferated to donor alloantigen *in vivo*. Furthermore, chimeric mice bearing intestinal allografts were capable of rejecting subsequently placed donor-strain skin grafts. These data suggest that although long-term allograft survival occurs in the absence of acute or chronic rejection, recipient mice are not completely unresponsive to donor alloantigens. When intestinal transplantation was performed at the time of initial bone marrow infusion (initiation of the chimerism protocol), most recipients failed to develop chimerism and promptly rejected the intestinal allograft. Although this is the most effective protocol that we have tested using this stringent model of transplantation, our observations suggest that modifications will be necessary before it can be reliably applied to the transplantation of highly immunogeneic organs like the intestine.

Key words: Bone marrow transplantation, chimerism, costimulatory blockade, intestinal transplantation

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Introduction

Intestinal transplantation is now recognized as definitive therapy for selected patients with intestinal failure. Despite the increased number of intestinal transplants performed, the outcome of intestinal transplantation remains inferior to that associated with the transplantation of other organs (1). This is mainly because of the greater immunogenicity of intestinal allografts and the associated increased frequency and severity of rejection (2,3). In addition to the problem of rejection itself, the relatively greater amount of immunosuppression required to prevent or control rejection following intestinal transplantation results in significantly increased rates of infection and post transplant lymphoproliferative disease (PTLD) relative to other transplanted organs (4–6).

The broader application of intestinal transplantation awaits the development of more effective and less toxic immunosuppressive regimens. In this regard, immunosuppressive strategies that promote donor-specific tolerance may offer particular benefits to patients undergoing intestinal transplantation. We have previously shown that agents including anti-CD4 mAb, anti-CD40L mAb, anti-LFA1, anti-B7.1 and anti-B7.2 mAb, and CTLA4Ig that promote tolerance and/or long-term survival of allografts in other transplant models fail to produce the same effect in the murine model of intestinal transplantation (7–9) (K. A. Newell, unpublished observations). Of the currently available approaches to tolerance induction, combined donor bone marrow and organ transplantation affords the advantage of inducing a robust tolerance to allografts in a number of experimental models. It was first recognized that hematopoietic chimerism was associated with donor-specific tolerance five decades ago (10,11). In 1955 Main and Prehn demonstrated that donor-specific tolerance could be acquired by infusing bone marrow cells into lethally irradiated adults (12). Concerns about infectious complications in fully allogeneic chimeras prompted investigators to design strategies for inducing mixed allogeneic chimerism (13). Subsequently protocols were designed that replaced the need for lethal irradiation with conditioning regimens that utilized anti-T-cell antibodies, low-dose TBI, and thymic irradiation (14) or costimulatory blockade combined with infusion of very large doses of donor bone marrow (200×10^6 BM cells/mouse) (15,16). However, persisting concerns about the long-term consequences of irradiating transplant recipients and the difficulty of

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obtaining the number of bone marrow cells required for the 'mega-dose' protocols may limit the clinical application of these approaches. Recently we have reported a regimen that completely replaces radiation as a conditioning agent with the alkylating agent busulfan. Busulfan preferentially depletes early hematopoietic stem cells without significantly affecting the number of mature circulating leukocytes in murine transplant models (17). Mice treated with busulfan, anti-CD40L mAb, CTLA4-Ig, and infused with two doses of donor bone marrow developed donor-specific tolerance as indicated by the indefinite survival of primary skin and heart allografts and acceptance of subsequently placed donor, but not third party skin grafts (17,18).

The aim of the current study was to determine the effect of this tolerance-inducing strategy on the outcome of intestinal transplants in mice. The results of our studies demonstrate that the infusion of donor bone marrow together with busulfan and costimulation blockade induces hematopoietic chimerism and promotes the long-term survival of intestinal allografts transplanted into mice that have completed the treatment regimen. This long-term survival is associated with donor-specific hyporesponsiveness *in vitro* and deletion of donor-reactive T cells *in vivo*. Interestingly, mice bearing long-term surviving intestinal allografts displayed significant prolongation of subsequently placed donor-strain skin grafts but were not tolerant by the strictest definition in that most donor-strain skin grafts were eventually rejected. Finally, unlike results obtained using skin and heart transplant models, most intestinal allografts placed on the initial day that the tolerizing regimen was begun were promptly rejected. These results provide a cautionary note and demonstrate that in its current form this approach to tolerance induction may not be clinically applicable for highly immunogenic organ allografts.

Materials and Methods

Mice

Adult male C57BL/6 (H-2^b), BALB/c (H-2^d), C3H/HeJ (H-2^k), and CB6F1/J (C57BL/6 × BALB/c, H-2^{bxd}) mice 6–8 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD). All mice were housed in specific pathogen-free conditions and in accordance with institutional guidelines. All studies were approved by and performed in compliance with the policies of the Institutional Animal Care and Use Committee of Emory University.

BM preparation and treatment regimens

Bone marrow was flushed from the tibiae, femurs, and humeri of BALB/c mice using sterile saline, needles, and syringes. Single-cell suspensions of harvested bone marrow were made. Red blood cells were lysed using a Trizma base ammonium chloride solution (Sigma, St. Louis, MO). The BM cells were resuspended at 2×10^7 cells/500 μL sterile saline and injected intravenously on days 0 and 6. Hamster antimouse CD40L mAb (MR1; Bioexpress, Lebanon, NH) and CTLA4-Ig (Bristol-Myers Squibb, Princeton, NJ) were administered on days 0, 2, 4, 6 (500 μg/dose i.p). A single 600 μg dose of busulfan (Orphan Medical Inc., Minnetonka, MN) was administered intraperitoneally on day 5.

Transplantation and histologic graft assessment

Intestinal transplantation was performed as described (7). Intestinal grafts were revascularized by anastomosing the portal vein to the recipient inferior vena cava and the superior mesenteric artery to the recipient infrarenal aorta. The jejunum was exteriorized as a stoma and the ileum was anastomosed to the side of the recipient jejunum. Recipient mice were sacrificed at predetermined time points in order to obtain tissue for histologic examination. These time points were initially chosen based upon our past experience with the model and clinical examination of recipient mice using criteria reported to be predictive of rejection in this model (19). Specimens for histologic assessment were fixed in 10% buffered formalin and embedded in paraffin. H&E-stained 3-μm sections were evaluated by a pathologist in a 'blinded' fashion. Rejection was graded according to the following definitions: 0, no rejection; 1, scattered apoptotic crypt cells; 2, focal crypt destruction; and 3, mucosal ulceration with or without transmural necrosis. Full thickness skin grafts (~1 cm^2) from the tails of donor mice were transplanted onto the dorsal thorax of recipient mice and secured with a Band-Aid for 7 days. Rejection was defined as the complete loss of viable epidermal graft tissue. Recipient death or graft loss within 7 days of the procedure was considered a technical failure.

Flow cytometric analysis

Peripheral blood was collected, the RBC lysed, and the remaining cells washed with a whole blood lysis kit (R & D Systems, Minneapolis, MN). Peripheral blood leukocytes (PBL) were then stained with the fluorochrome-conjugated Abs anti-CD3, anti-CD11b, anti-GR1, anti-B220, anti-H-$2K^d$, anti-H-$2K^b$, anti-Vβ11, anti-Vβ5.1/5.2, anti-Vβ8.1/8.2 (PharMingen, San Diego, CA), anti-CD4, anti-CD8 (Caltag, Burlingame, CA), or immunoglobulin isotype controls (PharMingen, Caltag). Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Donor chimerism expressed as a percentage that was calculated using the following formula: (H-$2K^{d+}$ cells/total gated cells) ×100.

Allogeneic mixed leukocyte reactions

Purified T cells (R & D systems, Minneapolis, MN) and dendritic cell-enriched transiently adherent splenocytes were used as responders and stimulators, respectively. A total of 10^4 irradiated (2000 rad, ^{137}Cs) stimulator cells were added to 10^5 responder cells in a final volume of 0.20 mL in 96-well round-bottom plates. Proliferation was measured by adding 1 μCi of [^{3}H] thymidine (Amersham, Arlington Heights, IL)/well after 72 h in culture. The cells were harvested 12–16 h later and counted on a beta-plate counter (LKB Instruments, Gaithersburg, MD). Results are the means of triplicate cultures.

Purification of T cells and T-cell subsets

Splenic $CD3^+$ T cells were purified by negative selection using commercially available murine T-cell isolation columns purchased from R & D Systems. Isolations were performed according to the manufacturer's instructions and resulted in >90% purity as assessed by flow cytometry.

In vivo proliferation assay

Briefly, spleens were harvested and a single-cell suspension was prepared in PBS. Red blood cells were lysed by hypotonic shock. 20×10^6/mL purified T cells (R & D systems) and were then labeled with CFSE (Molecular Probes, Portland, OR) at a final concentration of 10 μM in PBS at room temperature for 10 min Cell labeling was terminated by addition equal volume of FCS for 1 min. Cells were then washed twice in PBS before injection. Each CB6F1 mouse then received 30×10^6 CFSE-labeled cells via the penile vein. After 66 h, splenocytes were harvested from the recipients, and single-cell suspension was prepared after RBC lysis. Cells were stained with PE-conjugated H-$2K^d$ and anti-CD4 (PharMingen) and anti-CD8 (Caltag). Proliferation of CFSE-labeled donor T cells was analyzed by flow cytometry, as above.

Statistical analysis

Rejection grades were compared using the Kruskal–Wallis test for samples from multiple groups and the Mann–Whitney *U*-test for samples from two groups. Continuous variables were compared using the unpaired *t*-test with Welch correction. Calculations were performed using InStat version 2 (GraphPad, San Diego, CA). $p < 0.05$ was considered statistically significant.

Results

Costimulation blockade, busulfan, and donor bone marrow infusion result in multilineage hematopoietic chimerism and promote the long-term acceptance of intestinal allografts. Initial experiments were conducted to confirm that the regimen consisting of costimulation blockade utilizing anti-CD40L mAb, CTLA4-Ig, busulfan, and two infusions of donor bone marrow cells induced multilineage hematopoietic chimerism. Chimerism was initially detected 3–4 weeks after the first infusion of bone marrow cells and was first noted in the B-cell and macrophage compartments (data not shown). Donor granulocytes and T cells were first detected at slightly later time points (data not shown). Having confirmed that this strategy induced chimerism, we sought to determine the effect of this treatment on the survival of intestinal allografts. Following completion of the 6-day treatment regimen, mice were allowed to recover for 8–14 days before undergoing intestinal transplantation. It should be noted that because this is a heterotopic transplant model, the survival of recipient mice is not necessarily dependent upon the survival of the intestinal graft. Thus, in order to determine the status of the graft, recipients were sacrificed at predetermined time points, allowing the grafts to be assessed by both their gross and histologic appearance. As shown in Figure 1(A) the majority of BALB/c intestines transplanted into chimeric C57BL/6 recipients survived long-term. Mild rejection was noted in two of six mice at early time points (50–60 days). Two of seven mice developed severe rejection between 92 and 100 days. Histologic evaluation of the intestinal allografts of the five mice remaining at this later time point revealed normal histology remarkable for the absence of both acute and chronic rejection (Figure 1B). As shown in Figure 2, combined costimulation blockade, busulfan, and bone marrow infusion induced long-term hematopoietic chimerism in the majority of recipients with long-term surviving allografts (14 of 15 mice evaluated between 50 and 100 days). Of note although the majority of mice developed chimerism and experienced long-term survival of the intestinal allografts, the degree of chimerism attained varied significantly between recipients and did not reliably reach the high levels observed following transplantation of nonvascularized (17) or less immunogenic allografts (18). No increase in allograft survival was observed in mice that received costimulation blockade and infusion of bone marrow cells without busulfan relative to untreated recipients. This demonstrates that unlike some other transplant models (20,21) exposure to donor alloantigens under the cover of

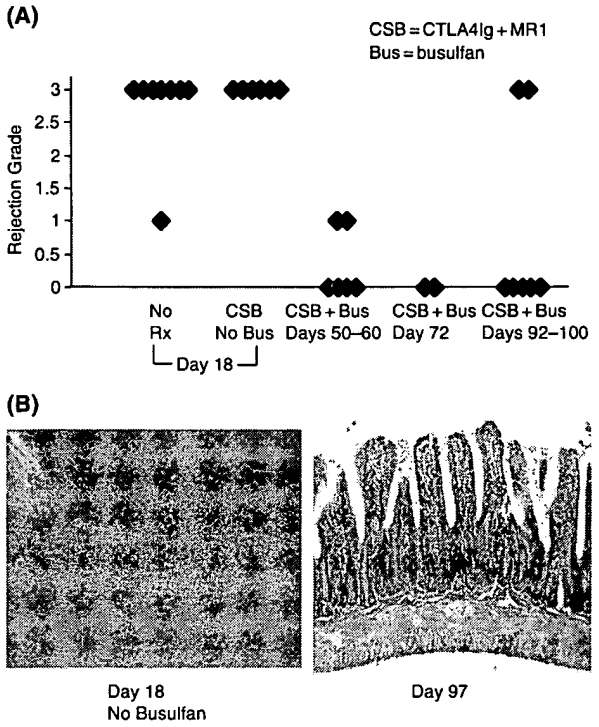

Figure 1: (A) Histologic scores of intestinal allografts from long-term surviving mice following combined costimulation blockade and infusion of donor bone marrow cells. Each symbol (◆) represents the score of an individual recipient. Scores of 0, 1, 2, and 3 denote normal histology, mild, moderate, and severe rejection, respectively. Allografts were assigned a score by a blinded pathologist using the criteria described in the Methods and Materials section. Three mice that survived more than 1 week were found dead of unknown causes precluding histologic graft assessment. (B) Representative H&E-stained sections of intestinal allografts from a recipient treated with combined costimulation blockade and bone marrow without busulfan (day 18) and a recipient treated with combined costimulation blockade, bone marrow, and busulfan (day 97).

costimulation blockade does not significantly inhibit the recipient immune response to intestinal allografts. Lastly, it should be noted that in addition to rejecting the intestinal allograft recipient mice that did not receive busulfan failed to display chimerism (data not shown).

Chimeric recipients of intestinal allografts display donor-specific hyporesponsiveness *in vitro* and delete T cells capable of recognizing MMTV antigens expressed by donor MHC molecules *in vivo*. We next sought to investigate the mechanism responsible for the long-term survival of intestinal allografts in recipients that had been treated using this chimerism-inducing protocol. As shown in Figure 3, T cells from chimeric mice bearing long-term surviving intestinal allografts displayed donor-specific hyporesponsiveness *in vitro*. T cells from the single nonchimeric recipient with a long-term surviving allograft

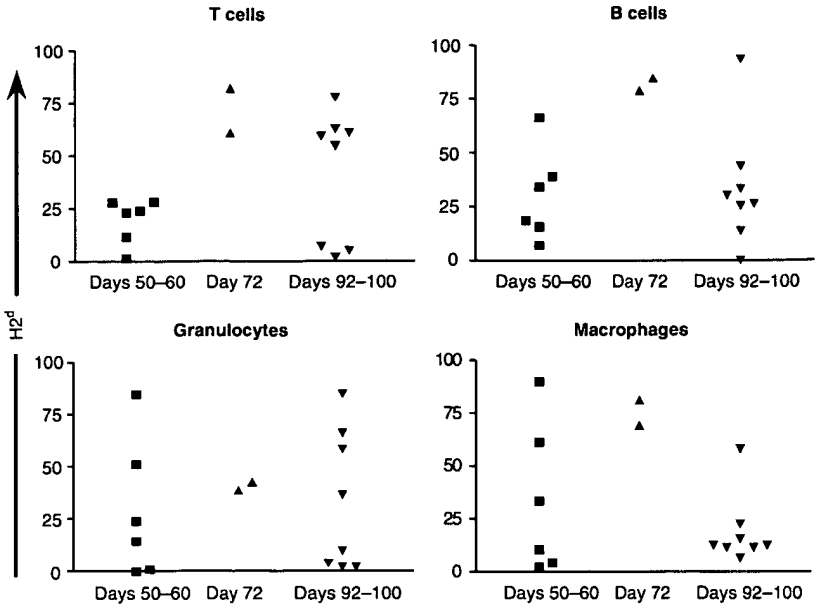

Figure 2: Percent of PBMC that express H-2K^d (donor MHC class I) as detected by flow cytometry. Results shown are for the last sample analyzed before sacrifice of the recipient. Days indicate time since initiation of the chimerism protocol. Each symbol (◆) represents the score of an individual recipient.

displayed a normal response to donor antigen. T cells from mice that were treated with only combined costimulation blockade and infusion of bone marrow cells before intestinal transplantation also proliferated well to donor alloantigen *in vitro*. Several mechanisms could underlie the lack of responsiveness to donor antigens including anergy of donor-reactive cells, the emergence of regulatory cells, and the deletion of donor-reactive T cells. In transplant models where allografts that express I-E are transplanted into recipients that do not express I-E, deletion of CD4+ T cells that express Vβ5 and Vβ11 has been used as a surrogate marker for the presence of T cells capable of responding to donor-strain alloantigens. We found that in C57BL/6 recipients (I-E$^-$) of BALB/c (I-E$^+$) intestinal allografts the frequency of T cells that expressed Vβ5 or Vβ11 following treatment with chimerism-inducing regimen was reduced (1.4 ± 0.7 and 2.8 ± 0.5, $n = 6$) compared with naïve C57BL/6 mice (2.9 ± 0.1 and 4.8 ± 1.6, $n = 3$) (flow cytometric data for an individual mouse from each group is shown in Figure 4). This suggests that deletion contributes significantly to the donor-specific hyporesponsiveness and prolongation of allograft survival that occurs following treatment with this chimerism-inducing regimen. However, even at late time points the frequency of donor-reactive Vα5$^+$ and Vα11$^+$ T cells in chimeric mice bearing BALB/c intestinal grafts had not fallen to the frequencies observed in naïve BALB/c mice (0.2 ± 0.1 and 1.0 ± 0.5, $n = 3$) (Figure 4).

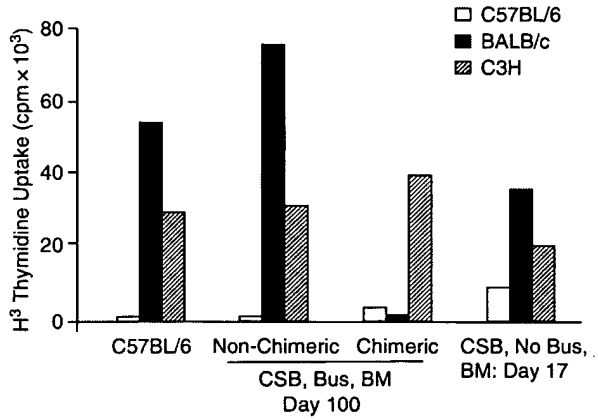

Figure 3: Results of a MLR comparing the proliferation of T cells from (A) a naïve, untransplanted mouse, (B) transplanted mouse that did not receive busulfan and was not chimeric, (C) transplanted mouse that did receive busulfan and was chimeric, and (D) the sole transplanted mouse that did receive busulfan and was not chimeric to self, donor, and third-party antigens. The result shown for the chimeric mouse with a long-term surviving allograft is representative of six recipients.

Recipient T cells that are capable of responding to donor alloantigens persist in chimeric recipients of intestinal allografts. Based on the hyporesponsiveness of recipient T cells to donor alloantigens *in vitro*, the deletion of donor-reactive T cells *in vivo*, and the prolonged survival of intestinal allografts in chimeric mice, we predicted that recipient T cells would also be hyporesponsive to donor antigen *in vivo*. As a first attempt to test this prediction T cells from untransplanted C57BL/6 mice chimeric with respect to BALB/c were studied using an *in vivo* proliferation assay. T cells from chimeric mice, naïve C57BL/6 mice, or naïve CB6F1 mice were labeled with CFSE and injected into CB6F1 mice. As predicted T cells from naïve C57BL/6 mice proliferated well in response to BALB/c alloantigens expressed by the CB6F1 recipient while

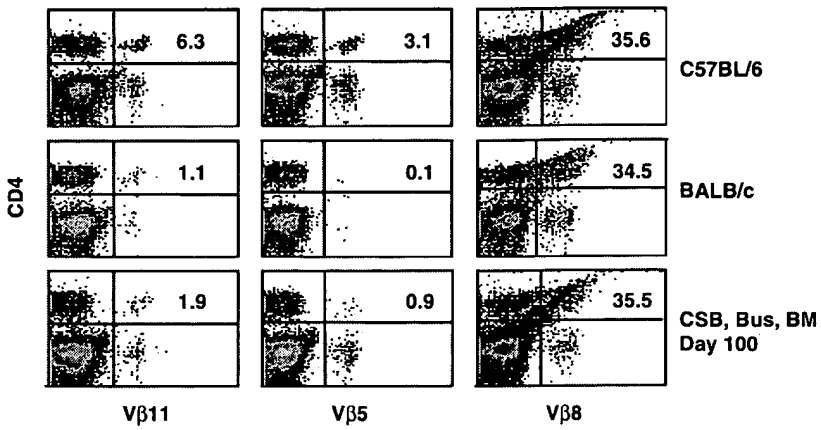

Figure 4: Representative histograms displaying the percentage of T cells expressing Vα5, Vα11, and Vα8.1–2 by T cells isolated from naïve C57BL/6 and BALB/c mice and from a day 100 chimeric C57BL/6 mouse bearing an intestinal allograft. The mean percentage Vα5, Vα11, and Vα8.1–2-positive T cells for each of these groups of mice is stated in the text.

syngeneic CB6F1 T cells did not proliferate when transferred into the F1 recipient (Figure 5). Surprisingly T cells from C57BL/6 mice chimeric with respect to BALB/c also proliferated *in vivo* in response to BALB/c alloantigen, indicating that T cells capable of responding to BALB/c alloantigens persist in chimeric mice. The finding that T cells from chimeric C57BL/6 recipients did not suppress the proliferation of T cells from naïve C57BL/6 recipients suggests that transferable regulatory T cells are not present at the time point examined (Figure 5). These findings raised the possibility that the chimeric mice were not fully tolerant.

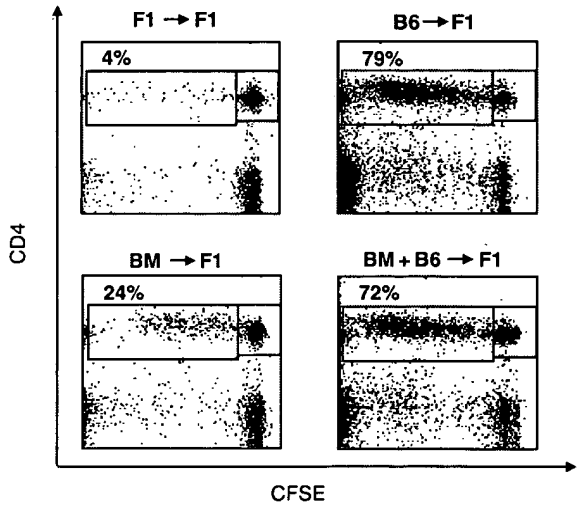

Figure 5: Proliferation of purified, CFSE-labeled T cells from naïve CB6F1/J mice, naïve C57BL/6 mice, chimeric C57BL/6 mice (32 days after the initial dose of combined costimulation blockade and donor bone marrow cells), or a one-to-one mixture of T cells from naïve and chimeric C57BL/6 mice following *in vitro* injection into CB6F1/J hosts. Histograms representative of the results obtained in two experiments (n = 5–6 mice per group total) are shown. In this experiment T cells were pooled from three untransplanted, chimeric C57BL/6 mice (the degree of chimerism detected within the B-cell compartment of these mice was 11%, 17%, and 21%). The percentage of proliferating cells is shown in parenthesis in the left upper quadrant.

In order to determine the consequences of the persistent BALB/c-reactive cells in chimeric mice, mice that had undergone transplantation of BALB/c intestines after receiving the chimerism-inducing regimen including infusion of BALB/c bone marrow cells were challenged with BALB/c (donor) and C3H/HeJ (third party) skin grafts. Grafting was performed at times ranging from 65 to 93 days after the initial infusion of BALB/c bone marrow. Seven of the eight mice were chimeric before skin grafting. As expected, third party skin allografts were promptly rejected. Surprisingly six of the eight skin grafts from BALB/c donors were rejected although their survival was significantly prolonged relative to third party grafts (Figure 6). Rejection of donor-strain skin allografts by chimeric mice bearing intestinal allografts did not result in the loss of chimerism (percent chimerism within the B-cell compartment was 16.8 ± 0.07% before skin grafting vs. 21.8 ± 0.12% after rejection of donor-strain skin grafts). It

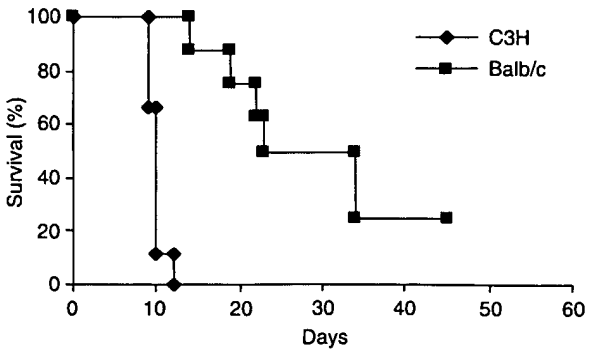

Figure 6: Survival of donor and third-party skin grafts placed on mice bearing intestinal allografts 65–93 days following initial treatment with donor bone marrow cells and combined costimulation blockade.

is also interesting to note that rejection of BALB/c skin grafts by chimeric mice did not invariably result in rejection of intestinal allografts (three mice rejected BALB/c skin grafts but not BALB/c intestinal grafts) although it should be noted that mice were only observed for short periods of time (20–45 days) following rejection of the skin grafts. Together, these finding demonstrate that donor-reactive cells persist in the chimeric mice and that these cells are capable of damaging grafts that express donor alloantigens.

Recipient mice that undergo intestinal transplantation before completion of the chimerism-inducing regimen reject intestinal allografts and fail to develop chimerism. Strategies of this type would be most easily applied clinically if the organ transplant could be performed at the same time that the chimerism-inducing regimen is initiated. We therefore performed a series of intestinal transplants on day 0 of the chimerism-inducing regimen (the day of initial bone marrow cell infusion and treatment with MR1 and CTLA4-Ig). Six of eight recipient mice that underwent intestinal transplantation on day 0 developed severe rejection by days 24–28 (rejection grade = three in all six mice). Furthermore, none of these six mice developed hematopoietic chimerism (data not shown). Of the two mice that did develop chimerism, depletion of donor-reactive Vα5 or Vα11 T cells was minimal 40 days following transplantation (data not shown). These results demonstrate that although prolonged survival and tolerance can be achieved in the mouse skin and heart transplant models when transplantation and chimerism induction begin at the same time (17,18), this is not the case for intestinal allografts in mice.

Discussion

Strategies using the infusion of donor bone marrow cells as a means of establishing mixed allogeneic hematopoietic chimerism and donor-specific tolerance to transplanted organs have been shown to be effective in both rodents and primates (14–17,22–25). Based on these studies trials have been initiated to test the efficacy of this approach in humans. The robust nature of this tolerance prompted us to test the effect of this strategy using a highly immunogeneic murine model of intestinal transplantation. We have previously examined a number of biological agents using this model including monoclonal antibodies specific for CD4, CD8, B7.1, B7.2, CD154, LFA-1, and membrane lymphotoxin and fusion proteins that bind B7, LIGHT, and 4–1BB. Of these agents only the anti-CD4 mAb, which modestly delayed acute rejection, and the anti-CD8 mAb, which inhibited acute but not chronic rejection, were at all effective in wild-type mice (26).

Against this background the finding that a chimerism-inducing strategy comprised of anti-CD154, CTLA4-Ig, busulfan, and donor bone marrow induces long-term survival of fully allogeneic intestinal grafts in the majority of recipients and that these allografts display no histologic features suggestive of chronic rejection represents a significant advance. This observation is consistent with a report that lethally irradiated rats rendered chimeric by the infusion of bone marrow cells failed to develop chronic rejection of intestinal allografts (27). Because in our experiments mice were not followed beyond 100 days, it is possible that chronic rejection could develop later. However, our previous experience with an anti-CD8 mAb demonstrated that chronic rejection of intestinal allografts can develop within 100 days.

While our data demonstrate that combined costimulatory blockade, busulfan, and donor bone marrow infusion is an effective approach for inducing the long-term survival of intestinal allografts, the mechanisms responsible for this effect have not been fully elucidated. Most groups investigating tolerance associated with chimerism-inducing strategies have reported deletion of donor-reactive CD4+ T cells using deletion of MMTV-reactive T cells as a surrogate for alloreactive T cells. It appears that both central and peripheral deletion contribute to this process (28,29). Using the same assay system we have also observed significant deletion of Vβ5+ and Vβ11+ MMTV-reactive CD4+ T cells. While the percentage of CD4+ T cells expressing Vβ5 and Vβ11 was markedly reduced relative to unmanipulated C57BL/6 mice, it was not reduced to the background level measured in unmanipulated BALB/c mice. This suggests that some donor-reactive cells could persist. If this is the case, additional mechanisms would be required to maintain donor-unresponsiveness. These additional mechanisms could include ignorance or anergy of the remaining undeleted cells and/or the development of regulatory T cells.

The need for additional mechanisms capable of controlling the alloresponse of mice treated with this chimerism-inducing regimen is most clearly demonstrated by comparing the outcome of intestinal transplants performed in recipients treated with combined costimulatory blockade, busulfan, and donor bone marrow with those that received only combined costimulatory blockade and donor bone marrow (no busulfan). The majority of recipients in the former group developed chimerism and experienced long-term allograft survival. While this was associated with deletion of donor-reactive cells, depletion was not maximal for at least 40–50 days. The finding that mice treated with only combined costimulation blockade and donor bone marrow all rejected the allograft by day 18 demonstrates that the immunosuppression provided by anti-CD154 and CTLA4-Ig with donor bone marrow is insufficient to prolong the survival of this highly immunogeneic allograft long enough to achieve significant depletion.

If costimulation blockade is not capable of prolonging survival beyond 18 days and deletion is not complete for several more weeks, how is allograft rejection prevented in these mice? Although at this time we can not distinguish definitively between ignorance, anergy, and regulation, the observation that depletion of CD4+ T cells, a potential

regulatory population, prevents the development of chimerism (17) (and data not shown for the intestinal transplant model) suggests that regulation may be important in this model. This hypothesis is consistent with other indirect evidence suggesting a role for regulation in tolerance induced by costimulation blockade and bone marrow infusion (29). Preliminary results from collaborative pilot experiments performed using the same chimerism strategy in a murine cardiac transplant model suggest that at least some recipients do develop regulatory populations of T cells (unpublished data, KAN, Charles Orosz and Alice Gaughan, Ohio State University, Columbus, OH). Although our studies performed using the CFSE *in vivo* proliferation assay failed to demonstrate a population of regulatory T cells, these studies do not exclude the existence of regulatory T cells in these chimeric mice. The *in vivo* CFSE assay only examined the effect of T cells from chimeric mice on the proliferation of alloreactive T cells at one time point. It remains possible that regulatory populations of cells develop in this model at different time points or that they exert their regulatory effect on events other than proliferation such as maturation, effector function, or cell trafficking. Substantially more investigation will be needed to confirm this finding and to determine its relative importance.

Regardless of the mechanism, using different transplant models we and others have reported that regimens that result in macrochimerism produce true tolerance to donor antigens. However, in the current set of experiments we observed prolonged but not indefinite survival of donor-strain skin allografts placed on chimeric mice bearing intestinal allografts. This finding demonstrates that these chimeric mice retain donor-reactive cells that are capable of mediating destruction of allografts expressing donor alloantigens. Therefore these mice are not tolerant by the strictest definition. This observation is consistent with a recent report by Russell et al. in which chimeric mice that had accepted donor-strain skin allografts developed chronic rejection of subsequently placed donor-strain heart allografts (30). The mechanisms underlying this 'split' tolerance remain uncertain. Based on the observation that untransplanted mice rendered chimeric using this protocol accept donor-strain skin grafts placed weeks to months after receiving the chimerism-inducing regimen (17), we do not believe that this finding is a consequence of skin-specific antigens. One potential explanation is that a recently placed nonvascularized skin graft may be more sensitive to a weak ongoing immune response than is an engrafted, vascularized organ. This model would be consistent with the observation that a small number of donor-reactive cells persist in mice following treatment with the chimerism protocol. A second potential explanation is that different types of organs or tissues display a differential susceptibility to injury by a given immunologic mechanism such as has recently been demonstrated for cardiac and skin allografts in mice (31). Although it is unexplained, this observation has important implications for the clinical application of this tolerance strategy.

Although it should be emphasized that the regimen consisting of combined costimulatory blockade, bone marrow infusion, and busulfan results in the best long-term allograft survival of any combination of agents that we have tested in the highly immunogeneic murine intestinal transplant model, our data suggest a potential problem that may affect the clinical implementation of this strategy. We observed that this strategy most often fails to promote chimerism and long-term allograft survival if intestinal transplantation is performed at the time of the initial bone marrow cell infusion. As this scenario reflects how this approach would be applied to humans undergoing transplantation with organs from cadaveric donors, this is a significant limitation. Interestingly, both skin and heart allografts transplanted in mice at the time the chimerism regimen is initiated survive long-term (17,18). We believe that this difference is a consequence of the greater immunogenicity of the intestine compared with the heart or skin. Unlike our initial work, which demonstrated that anti-CD154 and CTLA4-Ig significantly prolonged the survival of skin and heart allografts (32), our subsequent studies as well as data presented in Figure 1(A) show that these agents do not prolong the survival of intestinal allografts (9). Thus we believe that combined costimulatory blockade prolongs the survival of skin and heart allografts long enough for recipients to develop mechanisms that are capable of preventing rejection such as deletion or regulation. In contrast, all intestinal allograft recipients treated with these agents developed severe rejection by day 18; a period of time that is insufficient for deletion to have occurred and appears to also be inadequate for other mechanisms such as regulation to have developed. This line of reasoning suggests that more potent immunosuppressive agents that are compatible with the development of tolerance will need to be identified if bone marrow-based regimens of this type are to be successfully applied to the transplantation of highly immunogeneic organs like the intestine.

In conclusion, while our data demonstrate that this approach can result in the long-term survival of intestinal allografts in some settings, they also demonstrate potential limitations of this regimen such as the high frequency of failure when intestines are transplanted at the initiation of the chimerism regimen and persistence of donor-reactive cells in chimeric recipients. Approaches that overcome these limitations should allow the broader application of this regimen to clinical transplantation. Lastly, our data suggest that mechanisms other than deletion may contribute to the effectiveness of this regimen.

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References

1. Grant D. Intestinal transplantation. report of the international registry. Intestinal Transplant Registry. Transplantation 1999; 67: 1061–1064.
2. Sudan DK, Kaufman SS, Shaw BW Jr et al. Isolated intestinal transplantation for intestinal failure. Am J Gastroenterol 2000; 95: 1506–1515.
3. Farmer DG, McDiarmid SV, Yersiz H et al. Outcome after intestinal transplantation: results from one center's 9-year experience; discussion 1031–2. Arch Surg 2001; 136: 1027–1031.
4. Abu-Elmagd K, Reyes J, Bond G et al. Clinical intestinal transplantation: a decade of experience at a single center. Ann Surg 2001; 234: 404–417.
5. Nishida S, Levi D, Kato T et al. Ninety-five cases of intestinal transplantation at the University of Miami. J Gastrointest Surg 2002; 6: 233–239.
6. Fishbein T, Kaufman S, Schiano T et al. Intestinal and multiorgan transplantation: the Mount Sinai experience. Transplant Proc 2002; 34: 891–892.
7. Newell KA, He G, Hart J, Thistlethwaite JR Jr. Treatment with either anti-CD4 or anti-CD8 monoclonal antibodies blocks alpha-beta T cell-mediated rejection of intestinal allografts in mice. Transplantation 1997; 64: 959–965.
8. Newell KA, He G, Guo Z et al. Cutting edge: blockade of the CD28/B7 costimulatory pathway inhibits intestinal allograft rejection mediated by CD4+ but not CD8+ T cells. J Immunol 1999; 163: 2358–2362.
9. Guo Z, Meng L, Kim O et al. CD8 T cell-mediated rejection of intestinal allografts is resistant to inhibition of the CD40/CD154 costimulatory pathway. Transplantation 2001; 71: 1351–1354.
10. Owen RD. Immunogeneic consequences of vascular anastomoses between bovine twins. Science 1945; 102: 400–401.
11. Billingham RE, Brent L, Medawar PB. 'Actively acquired tolerance' of foreign cells. Nature 1953; 172: 603–606.
12. Main JM, Prehn RT. Successful skin homografts after the administration of high dosage X radiation and homologous bon marrow. J Natl Cancer Institute 1955; 15: 1023–1028.
13. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. Nature 1984; 307: 168–170.
14. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. J Exp Med 1989; 169: 493–502.
15. Wekerle T, Kurtz J, Ito H et al. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. Nat Med 2000; 6: 464–469.
16. Durham MM, Bingaman AW, Adams AB et al. Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. J Immunol 2000; 165: 1–4.
17. Adams AB, Durham MM, Kean L et al. Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. J Immunol 2001; 167: 1103–1111.
18. Shirasugi N, Adams AB, Durham MM et al. Prevention of chronic rejection in murine cardiac allografts: a comparison of chimerism- and nonchimerism-inducing costimulation blockade-based tolerance induction regimens. J Immunol 2002; 169: 2677–2684.
19. Cagiannos C, Zhong R, Zang Z et al. Effect of major histocompatibility complex expression on murine intestinal graft survival. Transplantation 1998; 66: 1369–1374.
20. Parker DC, Greiner DL, Phillips NE et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. Proc Natl Acad Sci U S A 1995; 92: 9560–9564.
21. Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. Am J Transplant 2002; 2: 501–509.
22. Tomita Y, Sachs DH, Khan A, Sykes M. Additional monoclonal antibody (mAB) injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mABs and 3-Gy whole body irradiation. Transplantation 1996; 61: 469–477.
23. Wekerle T, Sayegh MH, Ito H et al. Anti-CD154 or CTLA4Ig obviates the need for thymic irradiation in a non- myeloablative conditioning regimen for the induction of mixed hematopoietic chimerism and tolerance. Transplantation 1999; 68: 1348–1355.
24. Kawai T, Cosimi AB, Colvin RB et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. Transplantation 1995; 59: 256–262.
25. Kimikawa M, Sachs DH, Colvin RB, Bartholomew A, Kawai T, Cosimi AB. Modifications of the conditioning regimen for achieving mixed chimerism and donor-specific tolerance in cynomolgus monkeys. Transplantation 1997; 64: 709–716.
26. He G, Hart J, Kim OS et al. The role of CD8 and CD4 T cells in intestinal allograft rejection: a comparison of monoclonal antibody-treated and knockout mice. Transplantation 1999; 67: 131–137.
27. Orloff SL, Yin Q, Corless CL, Orloff MS, Rabkin JM, Wagner CR. Tolerance induced by bone marrow chimerism prevents transplant vascular sclerosis in a rat model of small bowel transplant chronic rejection. Transplantation 2000; 69: 1295–1303.
28. Wekerle T, Kurtz J, Sayegh M et al. Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. J Immunol 2001; 166: 2311–2316.
29. Wekerle T, Kurtz J, Bigenzahn S, Takeuchi Y, Sykes M. Mechanisms of transplant tolerance induction using costimulatory blockade. Curr Opin Immunol 2002; 14: 592.
30. Russell PS, Chase CM, Sykes M, Ito H, Shaffer J, Colvin RB. Tolerance, mixed chimerism, and chronic transplant arteriopathy. J Immunol 2001; 167: 5731–5740.
31. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8 (+) T cells mediate graft rejection via a distinct effector pathway. Nat Immunol 2002; 3: 844–851.
32. Larsen CP, Elwood ET, Alexander DZ et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 1996; 381: 434–438.